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## **Clinical Applications of Electron Microscopy in the Analysis of Collagenous Biomaterials**

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CLINICAL APPLICATIONS OF ELECTRON MICROSCOPY IN THE ANALYSIS OF COLLAGENOUS  
BIOMATERIALS

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Abstract

Scanning and transmission electron microscopy are of clinical value in assessing the interaction between biomaterials and ingrowing tissues. Ultrastructural information allows the clinician and biomaterials specialist to determine events occurring during wound healing and the biocompatibility of prosthetic devices. This paper reviews some of the experimental and clinical studies done in our laboratory on the use of natural and reconstituted collagen as replacements for connective tissues. Consideration is given to collagen flakes used for the treatment of dermal ulcers, a collagen fiber prosthesis used for tendon and ligament replacement, the effects of chemical preservatives on cartilage used for replacement of tissues during plastic surgery and the growth and orientation of nerve cells on reconstituted collagen fibers. Our results show that reconstituted collagen can be prepared into prosthetic devices which encourage cell attachment and orientation thereby facilitating healing of injured tissues. Furthermore chemical preservation of cartilaginous tissues kills chondrocytes resulting in eventual resorption by inflammatory cells.

Introduction

A variety of synthetic and natural polymers are used in prosthetic devices to replace damaged soft tissues (Goodship and Cooke, 1986; Weadock et al., 1987). Biodegradable and non-biodegradable synthetic polymers can be used as replacements for dermal (Doillon and Silver, 1986) and orthopaedic soft tissue structures (Alexander et al., 1986; Friedman et al., 1985; Salisbury et al., 1974). However, in these applications synthetic polymers often stimulate scar tissue at the expense of regeneration of normal tissue structure. In contrast natural collagenous biological materials have been used for centuries to repair tissues damaged by thermal, chemical and mechanical trauma (Pachence et al., 1987). These materials include amnion, placenta, skin, fasciae, free mucosa, heart valves, pericardium, nasal septum and other cartilages, tendons and ligaments (Pachence et al., 1987).

Natural collagenous tissues are used to replace heart valves, skin, tendons and ligaments. Prior to implantation these tissues are normally treated with glutaraldehyde for crosslinking and to decrease the antigenic response (Chvapil et al., 1973; McMaster et al., 1976). In some cases glutaraldehyde treatment is accompanied by additional tissue reactivity (Chvapil et al., 1983).

Purified collagen extracted from a variety of connective tissues has been used in the development of tissue replacements. Type I collagen is readily extracted from the skin and tendons of bovine tissues and reconstituted into a variety of physical forms that are stabilized by crosslinking (Kato et al., 1988a; Pachence et al., 1987). Improved cell replication and connective tissue deposition occurs within reconstituted collagen matrices thereby enhancing the healing of chronic ulcers (Doillon et al., 1988).

The purpose of this paper is to review the ultrastructural work performed

KEY WORDS: Scanning Electron Microscopy, Transmission Electron Microscopy, Collagen flakes, Collagen fibers, Nasal septum, Tendon prosthesis, Neuron.

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in this laboratory on natural and reconstituted collagenous materials used to replace and/or repair soft connective tissues found in skin, tendon/ligament, cartilage and nerve.

Clinical Observations On The  
Use Of Collagenous Materials To Treat  
Chronic Human Skin Ulcers

Loss of the epidermis and dermis is associated with prolonged bed rest and the reduction of blood supply to the skin. This occurs in the elderly population as well as in patients that have spinal cord injuries. Dermal ulcers frequently develop where bony prominences compress areas of the skin on the hips, heels and sacrum. In some cases skin ulcers can ultimately lead to exposure of muscle, tendon and bone (Silver et al., 1988). Our laboratory has developed a porous reconstituted type I collagen sponge in the form of flakes (see figure 1a) (Doillon et al., 1988; Silver et al., 1988) which is used as a wound dressing for treating skin ulcers. The flakes measure 2-5 mm in width and have pore diameters of 50 to 250  $\mu$ m and fiber diameters of 5 to 10  $\mu$ m (Doillon et al., 1986). Collagen flakes are preferred over collagen sponge or gel for treating ulcers because of their ease of handling which includes both application and removal from the wound. Their greater surface area increases the amount of tissue fluid that is absorbed which makes them ideally suited for applying to deep skin ulcers. Results from our previous studies indicate that treatment of dermal ulcers using a porous reconstituted type I collagen sponge in the form of flakes (see figure 1a) (Doillon et al., 1988; Silver et al., 1988) results in a 50% decrease in the wound area after eight weeks of treatment (Silver et al., 1988). Comparatively, patients treated with collagen sponges had a slower rate of healing (Silver, et al., 1988).

The protocol used in these studies involved daily washing of each ulcer with 1% (w/v) hydrogen peroxide followed by sterile saline and daily packing with sterile collagen flakes (Silver et al., 1988). Collagen flakes were removed from the wound prior to washing the wounds with hydrogen peroxide and saline. The flakes were then fixed (at pH 7.4) in 2% glutaraldehyde (v/v) and 0.1 M sucrose buffered by 0.1 M cacodylate buffer containing 1.5 mM  $\text{CaCl}_2$ . The flakes were post-fixed in 1%  $\text{OsO}_4$  (w/v), dehydrated and either embedded in Spurr resin for transmission electron microscopy (TEM) or critical point dried and sputter coated with 40 nm of gold for scanning electron microscopy (SEM). Thin sections were

double stained in 10% (w/v; aq) uranyl acetate and lead citrate (Reynold, 1963) and examined at 80 kV on a Philips 420 TEM. Scanning electron microscopy samples were examined at 10 kV on an AMRAY 1400 equipped with a titanium sublimation pump.

SEM showed that the collagen flakes were covered by leukocytes (see figure 1b). TEM also showed infiltration of leukocytes and bacteria into the collagen flakes (see figures 1c, 1d and 1e). Leukocytes were frequently surrounded by amorphous collagen (see figure 1e) instead of the fibrillar striated collagen seen in the normal flake. The bacteria observed were always surrounded by amorphous collagen (see figure 1e).

It is well known that both bacteria and leukocytes synthesize and secrete collagenolytic enzymes that may lead to the digestion of the collagen flakes (see figures 1d and 1e). Collagen molecules that comprise the flakes may bind these enzymes and decrease the amount of free enzyme that causes tissue destruction at the base of the wound. Daily washing and removal of the flakes may accelerate healing by decreasing the collagenase concentration and by chemotaxis of inflammatory cells and connective tissue cells into the wound.

Collagen Fiber Prosthetic Tendon/Ligament

Tendons/ligaments damaged by mechanical injury are frequently repaired using autogenous tendon/ligament. Repair of tendon/ligament using sutures may generate scar tissue joining the tendon/ligament to surrounding structures limiting function. To limit scar tissue formation, replacement of tendon/ligament is achieved using biodegradable and non-biodegradable synthetic polymers, and tendon grafts from the knee (Goodship and Cooke, 1986).

Synthetic polymeric implants have a limited fatigue life time and ultimately fail (Goodship and Cooke, 1986) while glutaraldehyde-treated bovine tendon and cadaver grafts elongate with time and eventually do not support loads in the knee (Friedman et al., 1985). Studies involving induction of a "neo-tendon" using a carbon fiber scaffold suggest that tendon/ligament structures may be repaired through the controlled formation of organized scar tissue (Alexander et al., 1986; Goodship and Cooke, 1986; Jenkins et al., 1977); however, carbon fibers tend to fragment and eventually become scattered throughout the joint (Thomas et al., 1987; Aragona et al., 1981).

Our laboratory has been involved in the development of a reconstituted collagen fiber prosthesis that was coated

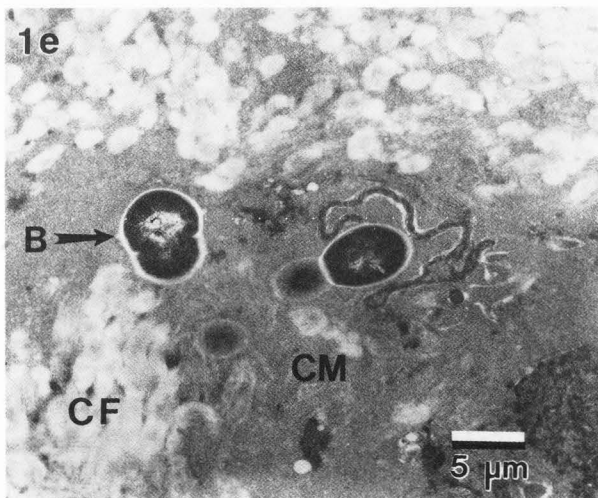
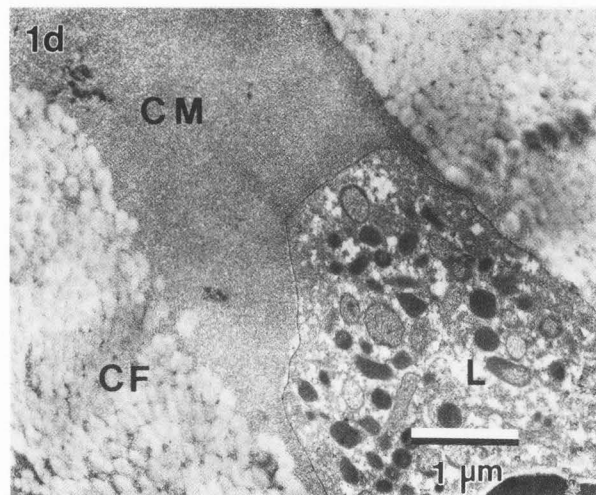
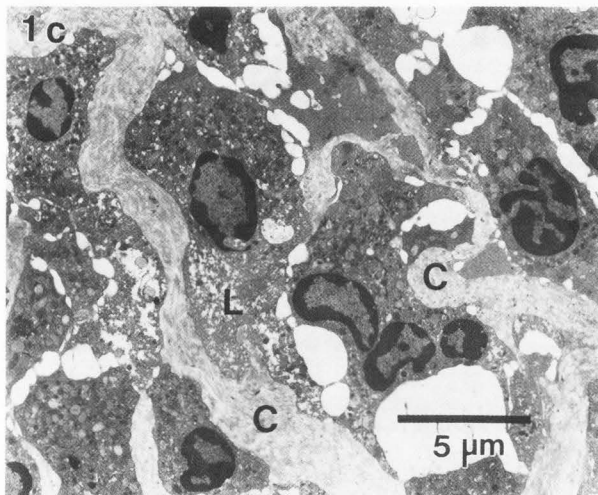
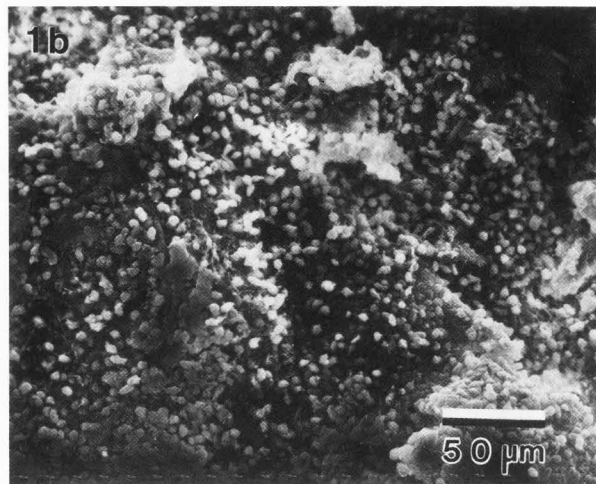
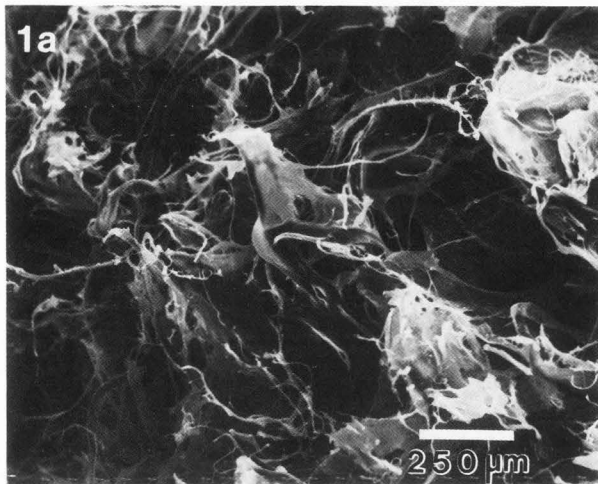


Figure 1. Scanning electron micrographs of reconstituted type I collagen in the form of flakes before (a) and after (b) removal from a skin ulcer. The surface of the flakes are covered with leukocytes and bacteria. Transmission electron micrograph of collagen flake (c) (shown in b) reveals leukocytes (L) in the pores of the collagen flakes (C). Leukocytes (L) (see d) are frequently surrounded by an amorphous matrix of collagen (CM). In e bacteria (B) are shown embedded in an amorphous matrix of collagen (CM). Note collagen fibrils (CF) in d and e.



with an unorganized collagen matrix (see figures 2a and 2b). Collagen fibers, 50 to 70  $\mu$ m in diameter, were produced by extrusion into a fiber formation buffer and crosslinked as described elsewhere (Garg and Silver, 1987). Crosslinked collagen fibers were coated with a 1% (w/v) collagen dispersion in HCl pH 2.0 and air dried overnight before sterilization by immersion for five hrs in a (2:1 v/v) solution of Alcide Exspor<sup>TM</sup> and phosphate buffer.

Under sterile conditions, the Achilles tendon was replaced in a white New Zealand rabbit (2.7 to 3.2 kg) with a collagen tendon/ligament prosthesis 6 cm long and 1 cm wide. One end of the prosthesis was weaved and sutured into the gastrocnemius muscle while the other end was weaved and sutured around the calcaneal junction. The incision was closed and three weeks postoperatively the tendon/ligament prosthesis was removed for ultrastructural studies. The prosthesis was fixed (pH 7.4, 4°C) in 1.5% glutaraldehyde (v/v)-4% paraformaldehyde (w/v) buffered by 0.1 M cacodylate containing 4 mM CaCl<sub>2</sub>. Post-fixation (pH 7.4, 4°C) in 1% OsO<sub>4</sub> (w/v) buffered by cacodylate, was followed by dehydration through a graded series of alcohols and embedding in Spurr epoxy resin. Thin sections were stained in 10% uranyl acetate (w/v;aq) and lead citrate (Reynolds, 1963) and examined at 80 kV in a Philips 420 TEM. For scanning electron microscopy, following dehydration samples were critical point dried and sputter coated with 40 nm of gold and examined at 5 kV in an AMRAY 1400 SEM equipped with a titanium sublimation pump.

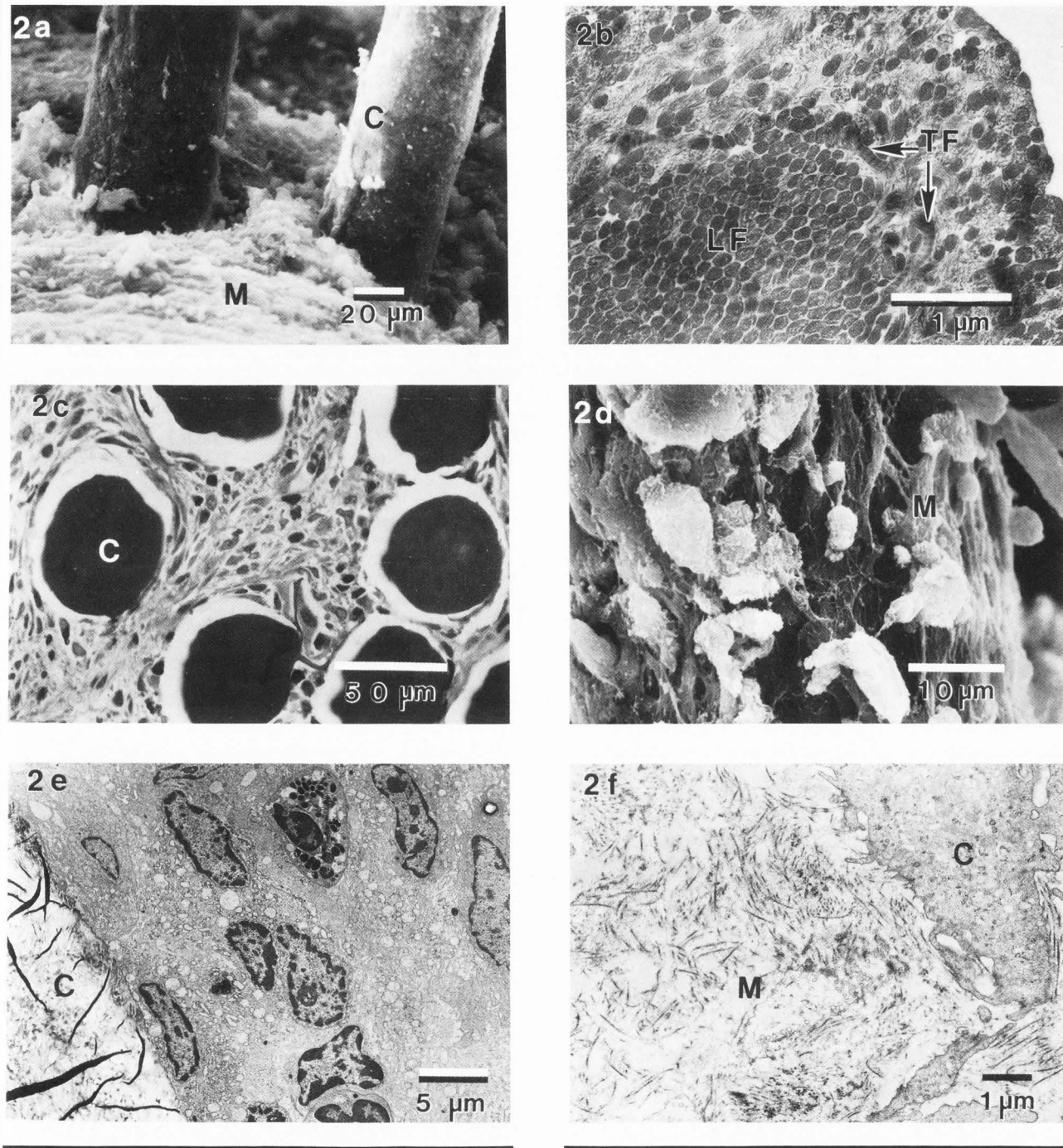
TEM observations were made on thin sections cut transversely from collagen fibers used to construct the tendon prosthesis. These results showed that the fibers consisted of a subpopulation of fibrils which were oriented in 2 directions (see figure 2b). Transverse fibrils ran perpendicular to the fiber axis and were clearly striated. Longitudinal fibers ran parallel to the fiber axis. These longitudinal fibers had a homogeneous amorphous appearance and were organized into periodic groups of fibril bundles. The fibers were held together by an amorphous matrix of non-striated collagen.

Animals were observed at 3, 10 and 20 weeks postoperatively. Thirty-one animals were examined by light microscopy. The results indicated that three weeks postoperatively newly deposited collagen was initially observed (see figures 2c and 2f) and collagen fiber degradation had begun (not shown). By 20 weeks the implant exhibited the crimped morphology of normal tendons based on observations using polarized light (not shown).

SEM and TEM observations showed that fibroblasts and other cells adhered to the collagen fibers of the prosthesis (see figures 2d and 2e). In some areas, cells were observed elongated along the axis of the prosthesis. The deposition of newly synthesized collagen was observed in the area around the fibrils (see figure 2f).

These results indicate that a collagenous implant may be acceptable for use as a replacement tendon. Invasion, cellular adherence to the fibers and the deposition of newly formed connective tissue around the implant occurs by three weeks. Simultaneous degradation of collagen fibers of the prosthesis by inflammatory cells suggests that rapid replacement of the existing implant with a newly synthesized neo-tendon occurs. A complete study to determine the rate of prosthesis degradation is currently in progress. The orientation of collagen deposited by fibroblasts may be a function of implant fibril diameter and subfibril suprastructure (Iversen et al., 1981; Ricci et al., 1984) and could ultimately determine the tensile strength and collagen organization of the newly synthesized tendon. Goodship and his colleagues (1985) report no differences in the extent of cellular infiltration and alignment for various synthetic implants. A crimp occurred in the collagen synthesized by fibroblasts in some of the prosthetic materials examined (Goodship et al., 1985).

Figure 2. Scanning electron micrograph (a) showing a collagen fiber tendon prosthesis composed of collagen fibers (C) embedded in a collagenous matrix (M). Transmission electron micrograph (b) of a collagen fiber in the tendon prosthesis cut in cross section showing a subpopulation of fibrils. Transverse fibrils (TF) run perpendicular to the fiber axis and longitudinal fibrils (LF) run parallel to the fiber axis. Longitudinal fibrils are frequently organized into groups of "fibril bundles". Light micrograph (c) showing connective tissue ingrowth around collagen fiber (C) of tendon prosthesis. Scanning electron micrograph (d) of a tendon explant showing a collagen fiber (not visible) covered with a myriad of cells embedded in their own extracellular matrix (M) or "neo-tendon". Note cells elongated parallel to fiber axis. Transmission electron micrographs of a tendon explant (e) showing a collagen fiber (C) surrounded by several cell types. Note some cells tightly adherent to the collagen fiber. In (f) a new collagen matrix (M) has been formed by invading cells (C) in area peripheral to the collagen fibers.



#### Cartilage Replacement/Preservation

Cartilage is used widely in reconstructive surgery (Donald, 1986; Gibson, 1977). Typical sources of cartilage for graft material include nasal septum, the 7th and 8th intercostal rib cartilages and conchal cartilage. It is a prerequisite that chondrocytes of graft material be alive or the tissue will be absorbed post-operatively

(Gibson, 1977; Peer, 1948). Live cartilage autografts are frequently unavailable, requiring the use of chemically or physically preserved allografts. Typical methods for the storage and preservation of cartilage include freeze drying, irradiation, storage in Merthiolate<sup>R</sup>, alcohol, Cialit<sup>R</sup>, formalin (Donald, 1986) and more recently Alcide Exspor<sup>TM</sup>. Cases of prolonged survival of stored grafts are

associated with the formation of a fibrous capsule which slows vascularization and degradation of the implant (Gibson and Davis, 1959).

Preservation of cartilage frequently causes chondrocyte death (Gibson, 1977). In a long term study of irradiated homologous cartilage used for the restoration of facial contour defects, progressive resorption of the grafts was reported with complete resorption anticipated (Welling et al., 1988). Resorption of irradiated and Methiolate<sup>R</sup> stored grafts has been reported in animal studies (Donald, 1986). During resorption of cartilage, fibrous tissue is laid down resulting in retention of the remodeled architecture. Hence, preservation methods which render cartilage non-viable should not be discounted since resorption does not always mean loss of contour restoration. A greater understanding of the effects of preservation on cartilage resorption is needed to improve methods of cartilage storage. In this study the effect of storage in Methiolate<sup>R</sup> and Alcide Exspor<sup>TM</sup>, on human nasal septum (HNS), has been assessed using SEM, TEM and the proteoglycan-specific electron stain quinolinic blue (Scott and Orford, 1981). Our goal is to understand the ultrastructural changes that occur after storage in Methiolate<sup>R</sup> and Alcide Exspor<sup>TM</sup>.

HNS removed during rhinoplasty or after storage at 4°C for 6 months in Methiolate<sup>R</sup> or Alcide Exspor<sup>TM</sup> was diced into 1 mm cubes while immersed in fixative (pH 7.4, 4°C). Either 1.5% glutaraldehyde (v/v)-4% paraformaldehyde (w/v) in 0.1 M cacodylate with 4 mM CaCl<sub>2</sub>, or 2.5% glutaraldehyde (v/v) in a critical electrolyte solution of 25mM Na acetate-0.3 M MgCl<sub>2</sub> with 0.05% quinolinic blue (w/v) (QB) were used (pH 7.5). Tissues were post-fixed (pH 7.4, 4°C) in 1% OsO<sub>4</sub> followed by block staining in 1% uranyl acetate (w/v). QB samples were only primary fixed. Tissues were dehydrated, infiltrated and embedded in Spurr resin for TEM. Thin sections were double stained in 10% (w/v; aq) uranyl acetate and lead citrate (Reynolds, 1963) or 1% (w/v; aq) sodium tungstate and 10% (w/v; aq) uranyl acetate for QB treated specimens. Samples were examined in a Philips 420 TEM. SEM samples were critical point dried and gold sputter coated with 40 nm of gold and examined at 20 kV in an AMRAY 1400 SEM equipped with a titanium sublimation pump.

TEM of untreated control HNS is seen in Figure 3a. The chondrocytes had long thin cytoplasmic fingers and contained numerous organelles. The cells were situated in thin lacunae and the surrounding matrix contained numerous

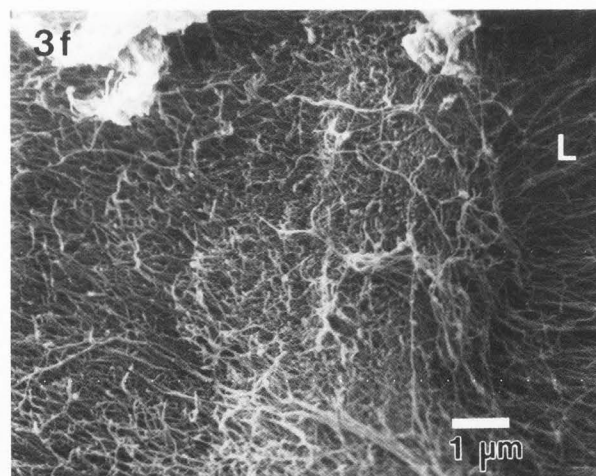
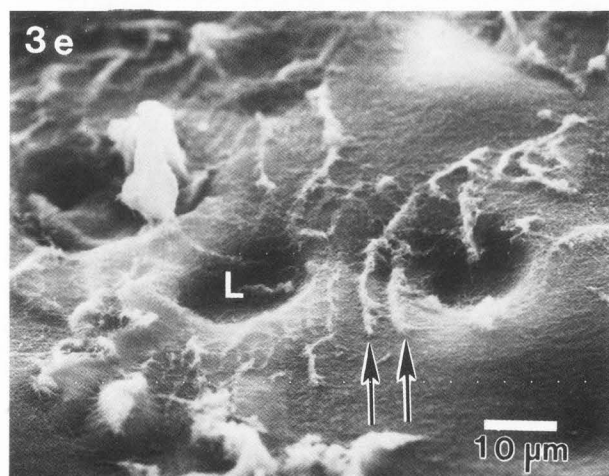
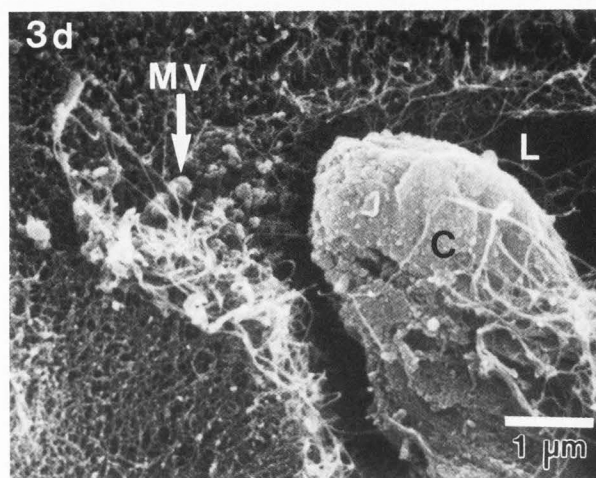
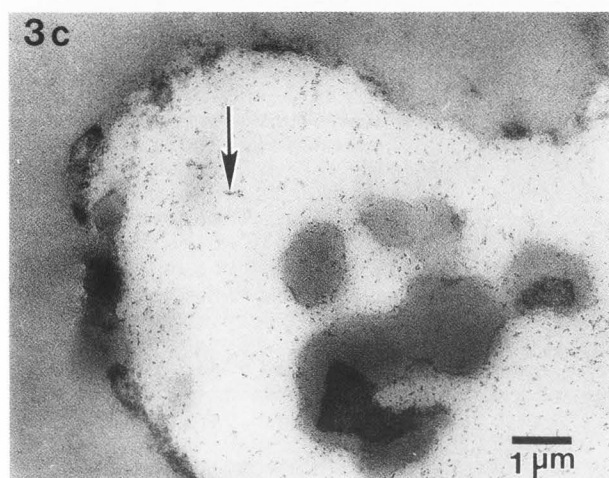
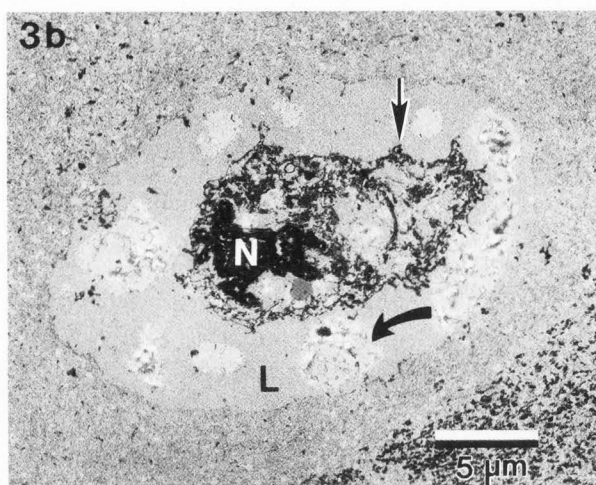
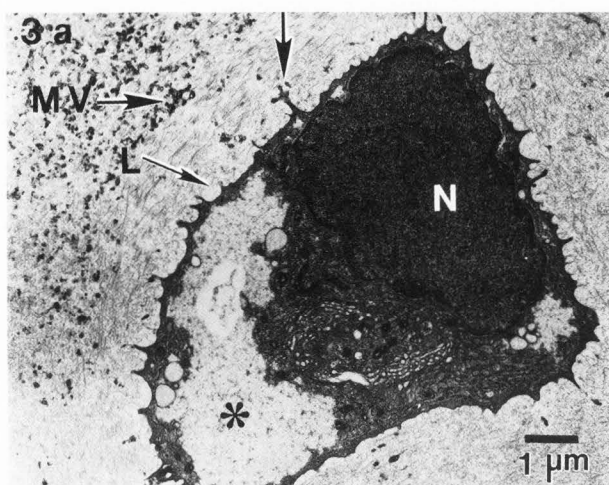
matrix vesicles. TEM of samples stored for 6 months in Alcide Exspor<sup>TM</sup> or Methiolate<sup>R</sup> (see figures 3b and 3c), showed chondrocytes which contained in their cytoplasm and pericellular lacunae, pools of amorphous material and proteoglycans as demonstrated by quinolinic blue positive staining. The cells had irregular plasma membranes with retracted and rounded cytoplasmic fingers, pyknotic nuclei, and proteoglycans. These cells were present throughout the cartilage. There was a disorganization of the collagen fibers composing the territorial matrix. Highly vesiculated matrix vesicles frequently contained proteoglycans.

SEM of untreated control HNS (see figure 3d) showed that chondrocytes were situated in enlarged lacunae (an artifact of specimen preparation). The matrix contained tightly packed "bristly" collagen fibers supporting numerous matrix vesicles. SEM of Alcide Exspor<sup>TM</sup> or Methiolate<sup>R</sup> HNS stored for 6 months (see figure 3e) indicated chondrocytes had fallen out of their lacunae which now appeared empty. The surface of the matrix was covered with numerous "cotton candy" wisps of flacid collagen fibers (see figure 3e). Matrix vesicles were not observed (see figure 3f).

Based on these observations we concluded that chondrocytes do not remain viable in Methiolate<sup>R</sup>, or Alcide Exspor<sup>TM</sup> solutions. Changes in proteoglycan distribution resulting from

Figure 3. Transmission electron micrograph of normal human nasal septum (a). Chondrocyte contains a nucleus with heterochromatin around periphery (N) and large areas of amorphous cytosol (\*). The cell has long narrow cytoplasmic fingers (arrow) which extend into an extremely thin lacuna (L). Matrix vesicles (MV) are embedded in the surrounding matrix. In contrast, a transmission electron micrograph of an Alcide Exspor<sup>TM</sup> treated human nasal septum (b) shows chondrocytes which have an irregular plasma membrane, retracted rounded cytoplasmic fingers (arrowhead) and pyknotic nucleus (N). The pericellular lacuna (L) is grossly enlarged and contains pools of amorphous material (curved arrow). After the matrix is stained with quinolinic blue, proteoglycans (arrow) are observed embedded in the pools of amorphous material (c). Scanning electron micrograph of human nasal septum (d) showing normal chondrocyte (C) within its lacuna (L). Note matrix vesicles (MV). Alcide Exspor<sup>TM</sup> treated septum (e,f) shows empty lacunae (L) with wisps of collagen which have formed on the matrix (arrow). Notice there are no matrix vesicles (f).





cartilage-proteoglycan detachment were also observed. The latter event may be responsible for the separation of chondrocytes from the enveloping territorial matrix since a pericellular proteoglycan coating cements the

chondrocyte plasmalemma to the surrounding territorial matrix (Schenk et al., 1986). SEM preparation exposed cells to a solution phase and they may be dislodged resulting in empty lacunae. The perturbation of the superstructure of

the matrix could allow matrix vesicles to fall out and collagen wisps to form. In TEM preparations, cells and vesicles were held in place by the surrounding matrix although they were probably loose.

It is likely the most suitable material for use as a graft material is a non-preserved allograft or autograft tissue. Cadaver cartilage stored for short periods (6 weeks) at 4-6°C may contain chondrocytes with sufficient metabolic activity to sustain the graft material (Gibson, 1977).

#### Collagen Fibers/Nerve Cell Growth

Injuries to the spinal cord or peripheral nerves can cause paralysis of limbs, lips or tongue (Colin and Donoff, 1984). The use of graft materials is one means of bridging severed nerves and expediting regeneration (Lundborg et al., 1982; Yannas and Orgill, 1985). There is little doubt that neurons will grow on a variety of collagen substrates (Iversen et al., 1981). Collagen is used successfully to encourage and support nerve regeneration (Colin and Donoff, 1984; DeLa Torre et al., 1984); however, collagen in the form of fibers has never been used. In addition there is little ultrastructural information about the orientation and adhesion of neurons growing on collagen. The use of collagen as a substrate for in vitro growth of isolated neurons has been investigated in our laboratory. The purpose of the experiments reported in this section is to develop a model system for the study of central nervous system injury and regeneration.

Fetal brains from 15-week old Sprague-Dawley rats were aseptically dissected and hippocampal neurons isolated using enzymatic digestion and centrifugation. Approximately 40,000 cells were plated on coverslips, containing collagen fibers, for 2 days at 37°C. The cells were fixed (pH 7.4, 4°C) in 2% glutaraldehyde (v/v) buffered by 0.1 M cacodylate containing 0.1 M sucrose. Post-fixation (pH 7.4) was performed in 1% OsO<sub>4</sub> (w/v) in 0.1 M cacodylate followed by block-staining in 1% uranyl acetate (w/v). For SEM, cells on cover slips were dehydrated, critical point dried and sputter coated with 400 Å of gold. Samples were examined at 5 or 10 kV on an Amray 1400 SEM equipped with a titanium sublimation pump.

Preliminary observations by light microscopy, of preparations from 5 separate animals showed that neurons grew on collagen fibers (see figure 4a). SEM studies from 2 animals confirmed these observations. Many neurons had long processes oriented along the axis of the fiber (see figure 4a). Swellings along the length of the neurite may represent

varicosities of synaptic vesicles. Small processes extending perpendicular to the fiber axis were observed sprouting from some neurites. Neurons frequently sent out collateral sprouts which adhered to a collagen fiber (see figure 4b). We counted 52 neurons growing on several different fibers from the 2 separate SEM animal preparations. Light microscopy observations of control cells grown on glass slides without collagen showed these cells had no preferential orientation.

Our tissue culture results demonstrate that collagen is a viable substrate for central nervous system cell adhesion and growth and may be very useful in the regeneration of injured peripheral and central nervous system neurons. The parallel orientation of the neurites along collagen fibers is similar to the results observed with other cell culture systems. For example, fibroblasts grown on synthetic fibers orient their long axis parallel to longitudinal surface striations (Ricci et al., 1984). Fibroblast-like cells cultured on grooved surfaces also orient themselves parallel to the grooves (Rovensky et al., 1975). Thus substrate geometry appears to play a role in cell orientation and is likely to involve some type of contact guidance or haptotaxis (Brunette, 1986). Similarly, substrate composition may affect cell adhesion. Epidermal cells from guinea pig skin grown on collagen have superior cell-to-cell and cell-to-substrate contacts compared to cells grown on plastic. This includes tight packing of neighboring cells and the formation of a basement membrane (Doillon et al., 1987; Doillon et al., 1988). Nerve cells in the present study appear plump and are attached to the collagen fibers. Fine collateral sprouts extended towards collagen fibers and this observation suggests some specificity of neurons for collagen fibers. Further experiments are needed to determine if nerve cell morphology is affected by tissue culture substrate composition. In other studies, the addition of hyaluronic acid and/or fibronectin can modulate collagen synthesis and cell replication (Doillon et al., 1987). Incorporation of other factors into collagen fibers may increase and influence nerve cell growth and orientation.

#### Summary

Collagenous tissues and reconstituted collagenous biomaterials are used to augment, replace or enhance healing of a variety of soft connective tissues. Preservation and sterilization of natural collagenous tissues results in cell death and eventual resorption. In contrast, implants formed from reconstituted collagen type I encourage

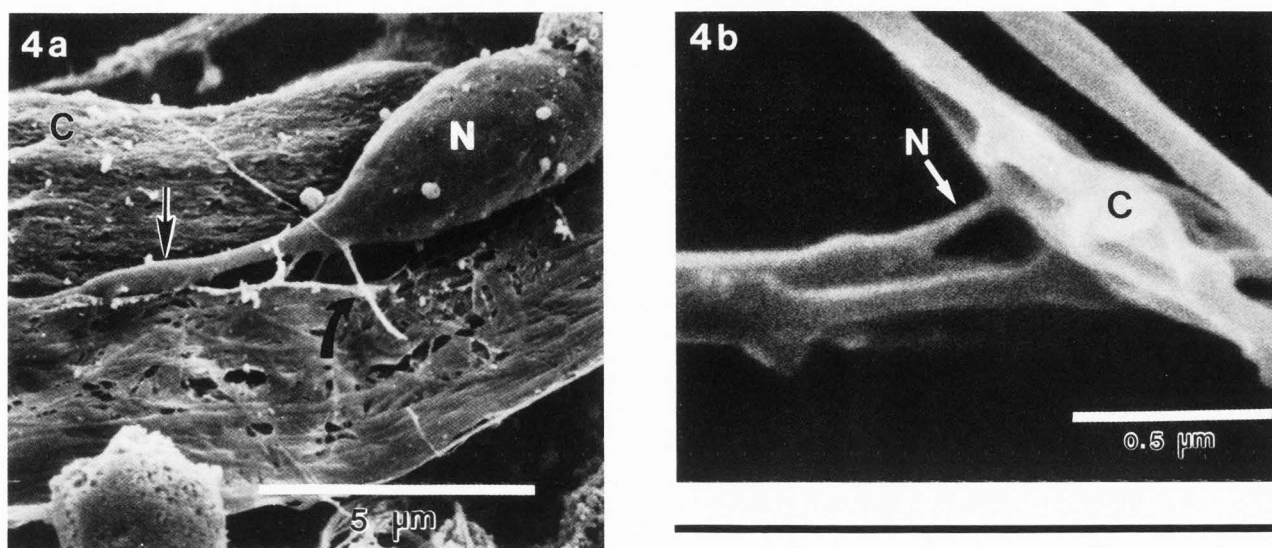


Figure 4. Scanning electron micrographs showing (a) neuron (N) attached to a collagen fiber (C). Note swelling (arrow) along neurite and collateral sprouts (curved arrow) originating from axon hillock. In figure b, a neuron (N) reaches for a collagen fibril (C).

cell infiltration and rapid replacement by living tissue. Seeding of collagenous implants with cells in culture may allow engineering of a wide variety of replacements for damaged or diseased tissues.

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#### Discussion With Reviewers

**M. Chvapil:** Why do the authors use Alcide Exspor<sup>TM</sup> sterilization? Does this indicate that 2.5 M cobalt radiation modifies collagen?

**Authors:** In this study, we used Alcide Exspor<sup>TM</sup> which is a cold sterilant. Literature on this product suggests that it can kill spores, viruses and bacteria within minutes of contact and this procedure can be done in any laboratory.

**A.E. Goodship:** In your work on the use of collagen flakes to treat chronic skin ulcers, the findings appear to be an inflammatory response with leukocyte infiltration and degradation of the collagen flakes. Was there any evidence of incorporation of the flakes or promotion of fibrosis within the dermal tissue?



**Authors:** We didn't take any tissue biopsies to determine if collagen flakes are incorporated into dermal tissue. This is an interesting possibility which can be evaluated in future animal studies. The difficulty and discomfort to patients of taking biopsies limits the use of human subjects.

**A.E. Goodship:** Did treatment with collagen flakes promote healing either by fibrous scar or by enhanced epithelialization to a greater extent than would have been seen without the application of such flakes?

**Authors:** Previously published data (Silver et al., 1988) from this laboratory shows that wounds treated with collagen flakes have a greater degree of wound closure as compared to control patients treated without flakes.

**A.E. Goodship:** It is suggested that daily washing and removal of the exogenous flakes may accelerate healing by decreasing the endogenous collagenase concentration and by chemotaxis of inflammatory cells and connective tissue cells into the wound. What quantitative evidence is there to support this hypothesis, what control procedures or alternative substances were used? Is there yet any evidence from your work to suggest that this material would provide an improved treatment for chronic skin ulcers?

**M. Chvapil:** I would like to see more justification and discussion as to why collagen flakes are preferable over sponge, gel or film in skin ulcers, as far as effectiveness and handling feasibility is concerned.

**Authors:** There is no quantitative evidence to support the hypothesis that collagen flakes soak up endogenous collagenase or serve as a chemotactic magnet for inflammatory cells. Preliminary data (not presented in this study) suggests that high levels of collagenase are present in chronic ulcers before healing initiates. Studies published elsewhere (Silver et al., 1988; Doillon et al., 1988) show that flakes heal ulcers more rapidly than non-treated ulcers.

**A.E. Goodship:** What were the mechanical properties of the constructed prosthesis. How were these mechanical tests performed and how did they relate to the characteristics of the normal tendon?

**Authors:** Mechanical testing results of the normal Achilles tendon and the explant is the topic of other manuscripts from this laboratory as well as of ongoing work (Kato et al., 1988a; Kato et al., 1988b).

**A.E. Goodship:** Perhaps the major objective in repair or replacement of skeletal tissues is restoration of normal mechanical integrity. How was this assessed in relation to the in vivo implantation of the prosthesis?

**Authors:** Studies are currently underway to assess the effect of the prosthesis on normal gait.

**A.E. Goodship:** What controls or comparisons were used for this study, either to study the material or functional biocompatibility benefits of reconstituted collagen in this application?

**Authors:** As described elsewhere controls consisted of devascularized autogenous Achilles tendon (Kato et al., 1988a).

**A.E. Goodship:** Did the two populations of collagen fibrils resulting from implantation show any difference in fibril diameters compared with those of the original prosthesis?

**Authors:** Fibril diameters of the explant were not measured. Based on our observations fibrils around the edges of the fibers of the prosthesis were noticeably smaller than those in the core of the fiber.

**A.E. Goodship:** The authors state that a collagenous (presumably reconstituted) implant may be acceptable for use as a replacement tendon. It is not clear to what extent such a device supersedes the use of autogenous grafts or synthetic fibrogenic implants in terms of induction of normal collagen or enhanced restoration of functional characteristics.

**Authors:** Synthetic implants have a limited fatigue life. There is less than optimum tissue ingrowth consequently when the device fails there is less neo-tendon to offer biomechanical support compared to the original tendon. A second surgery or procedure is required to remove, replace or repair the tendon prosthesis if it fragments. Based on our studies a collagenous tendon prosthesis induces neo-tendon formation which is significant by 20 weeks.

**A.E. Goodship:** Is there evidence that the clinical results of grafts stored in these agents reflect the findings of your ultrastructural studies, since the statement that these are common methods of storage would imply a reasonable clinical result, therefore are viable chondrocytes required for the incorporation of the graft? You appear to conclude that no method of medium to long term storage will provide a graft that can be sustained.

**Authors:** We believe no method of storage can be relied upon to provide a

consistently viable cartilage for use as a graft material. The reason for continued use of chemically or physically preserved cartilages in reconstructive surgery is lack of a better material. It is prerequisite that chondrocytes are alive for cartilage to remain viable (Gibson, 1977). Merthiolate<sup>R</sup> and irradiation kill chondrocytes, producing non-viable cartilage which will be resorbed (Hagerty et al., 1960; Donald, 1986; Welling et al., 1988).

J.L. Drummond: How many specimens were examined in this study and were the results consistent with your preliminary observations?

Authors: Although this is a pilot study we observed 5 preparations by light microscopy and 2 by SEM. In all cases there were neurons growing on collagen. We counted 52 neurons growing on collagen fibers in the 2 electron microscopy preparations. The goal of this study was to attract, orient and culture central nervous system neurons on collagen. Future studies will statistically analyze the effects of changes in surface chemistry and morphology on the above parameters.

D.W. Gregory: You have mentioned simultaneous degradation of collagen fibers. Can some evidence be presented for this?

Authors: Degradation of tendon prosthesis fibers has been well documented by this laboratory. This event is observed by light and electron microscopy as early as 3 weeks. By TEM collagen fibrils can be seen as small black dots that have separated from the body of the fiber. By SEM, fibrils which appear as fine threads, can be seen laying loosely upon the surface of the fiber body. By light microscopy, large portions of circular fibers are missing producing deformed fibers. Studies of the biodegradation of collagen fibers at the light and electron microscopic levels are ongoing and will appear elsewhere.